野口英世著　Journal of Experimental Medicine　所収論文

FURTHER STUDY ON THE CULTURAL CONDITIONS OF LEPTOSPIRA (SPIROCHÆTA) ICTEROHÆMORRHAGIÆ.

By HIDEYO NOGUCHI, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, February 20, 1918.)

The cultivation of *Leptospira (Spirochæta) icterohæmorrhagiæ*[1] is comparatively simple. It was first accomplished by Inada and his coworkers[2] by means of the method recommended by me for the cultivation of several varieties of blood spirochetes.[3] Later, various techniques for the isolation of this organism on artificial media were proposed by Ito and Matsuzaki,[4] Reiter,[5] Martin, Pettit, and Vaudremer,[6] and myself.[7] While all the methods appear to have given satisfactory results, there is no unanimity as to the best one to be followed in routine work. As far as I am aware, there has been no critical analysis of the conditions requisite for uniform success in obtaining a culture. I wish to report here some of the results of my study of the various strains from Asiatic, European, and American sources.

Necessity of Fresh Serum Constituents for the Growth of Leptospira icterohæmorrhagiæ.

My first cultures of the Japanese, European, and American strains of *Leptospira icterohæmorrhagiæ* were obtained by employing a medium containing about 1 part of normal rabbit serum and 2 parts of Ringer's solution, with the addition of an adequate amount of citrate plasma.[8] The rate of multiplication of the organism is faster at 37°C.

[1] Noguchi, H., *J. Exp. Med.*, 1918, xxvii, 575.

[2] Inada, R., Ido, Y., Hoki, R., Kaneko, R., and Ito, H., *J. Exp. Med.*, 1916, xxiii, 377.

[3] Noguchi, *J. Exp. Med.*, 1912, xvi, 199.

[4] Ito, T., and Matsuzaki, H., *J. Exp. Med.*, 1916, xxiii, 557.

[5] Reiter, H., *Deutsch. med. Woch.*, 1916, xlii, 1282.

[6] Martin, L., Pettit, A., and Vaudremer, A., *Compt. rend. Soc. biol.*, 1917, lxxx, 197.

[7] Noguchi, *J. Exp. Med.*, 1917, xxv, 755.

[8] About 0.5 part.

than at 25°C., but on the whole the first generation grows much more slowly than a later generation. It may be several days before growth is definitely ascertained.

The question may be raised as to what part of the serum is essential for the cultivation of the organism. For the purpose of determining this point, a portion of a mixture of rabbit serum 1 part, and Ringer's solution 3 parts, was heated to 60°C. for 30 minutes and another portion to 100°C. for 15 minutes. Unheated serum was used as control. It was found that heating to 100°C. for 15 minutes destroyed the nutrient value of the rabbit serum. Heating to 60°C. for 30 minutes reduced but did not destroy its cultural value as compared with the control. The nutrient principle of the serum, therefore, is closely associated with coagulable serum proteins. Filtration through the Berkefeld filter does not alter the cultural value of the serum medium.

Comparative Nutrient Value of Various Sera.

Not many animals are susceptible to the inoculation of *Leptospira icterohæmorrhagiæ*, and the guinea pig is the only animal in which the infection is almost invariably fatal. Rabbits are comparatively resistant, 1 to 2 cc. of a well growing pure culture being required to produce jaundice, whereas 0.000001 cc. of the same cultures may cause typical symptoms and death in a guinea pig. Dogs are more sensitive than rabbits, while cats, white rats, mice, and wild rats tolerate the infection and become carriers. A comparison of the suitability of various animal sera for purposes of cultivation of the organism is of practical as well as of biological interest.

Sheep Serum.—Of twelve different sheep sera, only four were found to be suitable, and in these the life of the organism was much shorter than in rabbit serum medium. A mixture of serum 1 part, Ringer's solution 3 parts, and 1.5 per cent agar 0.5 part was used. Undiluted sheep sera gave no better results, nor was the use of the citrate plasma from sheep advantageous.

Guinea Pig Serum.—Eight different lots of guinea pig sera were tested, each lot containing the sera from several animals, and good results were obtained in all. The sera were diluted three times with Ringer's solution and a small amount of agar or citrate plasma was

added. In this medium, however, the organism died out much sooner than in the rabbit serum medium.

Horse Serum.—Two out of four different horse sera proved to be very satisfactory, especially when used in a mixture of 1 part serum, 3 parts Ringer's solution, and 0.5 part 1.5 per cent agar. In this medium the culture survived for many weeks.

Calf Serum.—Only two calf sera were tested, but both gave a fairly good growth. A 1:4 dilution of serum with Ringer's solution was better than the undiluted serum. Martin, Pettit, and Vaudremer[6] recommend a 1:10 dilution of this serum as most suitable.

Goat Serum.—The only serum tested was very suitable when used in a mixture of 1 part serum, 3 parts Ringer's solution, and 0.5 part 1.5 per cent agar. The undiluted serum did not give so good a growth.

Donkey Serum.—The one available specimen proved totally unsuitable.

Pig Serum.—Two pig sera were tested, but the culture failed to grow in any concentration.

Rat Serum.—The sera from about twenty white rats were mixed and tested for their nutrient value. Diluted as well as undiluted sera were employed, but the results were negative.

Human Serum.—Five specimens which had been collected many months previous to the time of testing proved to be without any nutrient value for the organism in question. Two other specimens, which were freshly collected[9] from syphilitic patients, were found to be fairly suitable when used in proportions of 1:1 and 1:3 with Ringer's solution. The culture was short lived, however, reaching its greatest growth in about 11 days at 37°C. and dying off during the following week. The growth of the culture in the rabbit serum control medium was still increasing when the other cultures died.

Ascitic Fluid.—Twenty different samples of ascitic fluid were tested. They were used undiluted and also in different dilutions with Ringer's solution, but up to the present time none has been found suitable for the cultivation of *Leptospira icterohæmorrhagiæ*.

[9] These specimens were obtained through the courtesy of Dr. David J. Kaliski.

Nutrient Value of Organ Emulsions.

In the later stages of infection *Leptospira icterohæmorrhagiæ* invades the visceral organs in enormous numbers, the liver and kidneys being principally involved. One might infer, therefore, that these organs contain an abundant quantity of the substances favorable for the life and multiplication of the organism, and that an emulsion of these organs would constitute an ideal culture medium. The experimental data, however, did not support this assumption.

Emulsions of approximately 5 per cent in Ringer's solution were prepared with the liver, kidney, spleen, heart muscle, and testicle of a normal rabbit and a normal guinea pig, killed by bleeding, and tested for their nutrient value as culture media. In order to make the conditions of the media as varied as possible, the emulsions were used in four different ways: in one set of tubes the emulsion was used alone and unheated, in the second it was heated to 60°C., in the third it was heated to 100°C., and in the fourth there was added agar amounting to 0.3 per cent. The mixture of rabbit serum, Ringer's solution, and citrate plasma and that of rabbit serum, Ringer's solution, and agar were used as control media.[7] In the media containing the organ emulsions no sign of growth of the spirochete was observed, while excellent cultures were obtained in the control media.

The organs of guinea pigs were just as unsuitable for the cultivation of the organism as those of rabbits. I was not unaware of the possible change in the reaction due to autolysis of the organ cells, or of the injurious effect which certain autolytic cleavage products might have, but the emulsions showed a weak alkaline reaction throughout the experiments.

When rabbit serum, in the proportion of approximately 25 per cent, was added to a number of the tubes containing the emulsions, the spirochete multiplied vigorously; therefore, the fact that no culture was obtained with pure organ emulsions must have been due to the absence of suitable nutrient substances for the organism.

Egg White and Egg Yolk as Culture Media.

The failure of various organ emulsions to serve as culture media turned my attention to the possibility of utilizing egg white and egg

yolk for the purpose. The white and yolk of an egg were separated and each was diluted with Ringer's solution in different proportions: 2.5 cc. + Ringer's solution 2.5 cc.; 1 cc. + Ringer's solution 4 cc.; 0.5 cc. + Ringer's solution 4.5 cc.; and 0.25 cc. + Ringer's solution 4.75 cc. In each instance one set of tubes was used in the fresh state and the other heated to 55°C. for 24 hours with a view to possible improvement of nutrient value. In none of the egg media was any culture obtained, nor did the addition of the rabbit serum enhance their nutrient value beyond that of the serum.

Concentration of the Serum in Culture Media.

The importance of the presence of serum for the successful growth of the spirochete having been demonstrated, the following experiments were undertaken in order to determine the influence of various

TABLE I.

Japanese strain.	37°C.		26°C.	
	7 days.	30 days.	7 days.	30 days.
Undiluted rabbit serum.....	+	+++	+	+++
50 per cent rabbit serum + Ringer's solution....	+	+++	+	+++
33 " " " " + " "	+	+++	+	+++
25 " " " " + " "	+	+++	+	+++
20 " " " " + " "	+	+++	+	+++
15 " " " " + " "	+	+++	+	+++
10 " " " " + " "	+	+++	++	+++
5 " " " " + " "	+	+	+	+++

European strain.	Cultures, 30 days at 26°C.	Proteins precipitable with 10 volumes of absolute alcohol.
33 per cent rabbit serum + Ringer's solution	+++	Copious coarse precipitate and opalescence.
20 " " " " + " "	++	Copious coarse precipitate and opalescence.
10 " " " " + " "	++	Minute granules and opalescence.
5 " " " " + " "	+	Opalescence.
2 " " " " + " "	−	"
1 " " " " + " "	−	Granular.
0.5 " " " " + " "	−	

TABLE I—*Concluded.*

American strains.	Cultures, 30 days at 26°C.		
	Strain 1	Strain 2	Strain 3
33 per cent rabbit serum + Ringer's solution...	+++	+++	+++
20 " " " " + " " ...	+++	+++	+++
10 " " " " + " " ...	++	+++	++
5 " " " " + " " ...	+	++	+
2 " " " " + " " ...	−	−	−
1 " " " " + " " ...	−	−	−
0.5 " " " " + " " ...	−	−	−

The above experiments show that a maximum growth may be obtained with all strains tested in a medium containing more than 20 per cent serum, while a 10 per cent serum medium may give as much growth, but only with certain strains. The growth is scanty in a 5 per cent serum solution, and in a medium containing 2 per cent or less there is no growth.

concentrations of serum upon the culture. Table I summarizes the results.

Influence of Diluents and of Salt Concentration upon the Culture.

The apparent indifference of the spirochete to salt constituents of the culture media was noticed from the beginning of the cultivation

TABLE II.

American strain No. 1	Cultures 30 days at 26° C.
Rabbit serum 1 cc. + 10 per cent sodium chloride 4 cc. = 8 per cent sodium chloride	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 2 cc. + water 2 cc. = 4 per cent sodium chloride	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 1 cc. + water 3 cc. = 2 per cent sodium chloride	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 0.5 cc. + water 3.5 cc. = 1 per cent sodium chloride	+++
Rabbit serum 1 cc. + 10 per cent sodium chloride 0.25 cc. + water 3.75 cc. = 0.5 per cent sodium chloride	+++
Rabbit serum 1 cc. + water 4 cc. = salt-free control	+++
" " 1 " + Ringer's solution 4 cc. = serum-Ringer's solution control	+++

experiments. Instead of Ringer's solution, a 0.9 per cent saline solution or distilled water could be used as a diluent. In fact sewer water and stagnant or ordinary tap water were found to be satisfactory diluents when previously rendered sterile by filtration or autoclaving. The organism displays great tolerance not only to various neutral salts or organic matter which are apt to be present in sewer or stagnant water, but also to an increasing concentration of sodium chloride. The relation of salt concentration to growth is shown in Table II.

There was no perceptible difference in the degree of growth of the organism in this experiment, or in its morphological features. The tonicity of the culture medium is apparently an unimportant factor.

Effect of Reaction upon the Culture.

Leptospira icterohæmorrhagiæ seems to be one of the most sensitive of the microorganisms to the reaction of the culture medium. A slight variation to acid or alkaline from a given optimum zone renders a medium totally unsuitable for the growth of the organism (Table III).

Considering the minuteness of the quantities of hydrochloric acid or sodium hydroxide which were added in these experiments, and the extent to which the reagents were finally diluted with serum and distilled water, one cannot fail to realize the great importance which the reaction of the culture medium must have in relation to the growth of the organism. Similar results were obtained with the Japanese and European strains. The first requisite to the successful cultivation of *Leptospira icterohæmorrhagiæ* appears to be an optimum reaction of the culture medium, which, in my experience, lies between a slight alkaline reaction and that resulting from subsequent multiple dilutions with indifferent diluents (distilled water, isotonic salt solution, Ringer's solution, etc.).

A considerable fluctuation was found by titration of the sera of several domestic animals. For example, 2 cc. of the sera of the sheep, donkey, ox, and pig, each mixed with 3 cc. of distilled water, required 0.4 cc. of 0.1 N hydrochloric acid to bring about a neutral reaction, and 0.6 cc. to cause distinct acidity and turbidity. Rabbit serum had a uniformly weaker reaction, only 0.2 cc. of 0.1 N hydro-

TABLE III.

American strain No. 1	Physical changes.	Reaction to litmus paper.	Result of cultivation at 30° C. for.	
			6 days.	18 days.
Rabbit serum 1 cc. + water 3 cc.	Clear.	Slight alkaline.	+++	++
" " 1 " + 1.5 per cent agar 0.5 cc.	"	Slight alkaline.	+++	++++
Addition of acid.				
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.1 cc.	Slight opalescence.	Neutral.	+	−
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.1 cc. + 1.5 per cent agar 0.5 cc.	Slight opalescence.	"	+	−
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.2 cc.	Many sandy precipitates on wall and bottom.	Trace of acid.	−	−
Rabbit serum 1 cc. + 0.1 N hydrochloric acid 0.2 cc. + 1.5 per cent agar 0.5 cc.	Slight opalescence.	Trace of acid.	+	−
Addition of alkali.				
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.1 cc.	Clear.	Distinct alkaline.	−	−
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.1 cc. + 1.5 per cent agar 0.5 cc.	"	Distinct alkaline.	−	−
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.2 cc.	"	Stronger alkaline.	−	−
Rabbit serum 1 cc. + 0.1 N sodium hydroxide 0.2 cc. + 1.5 per cent agar 0.5 cc.	"	Stronger alkaline.	−	−

chloric acid being required to produce a neutral, and 0.35 cc. an acid reaction. The reaction of horse serum lay between that of the rabbit and that of the other animals. The fact that some of the latter sera showed a better nutrient value in dilution may be explained by the reduction of native alkalinity through dilution.

Oxygen Requirement of the Culture.

At the beginning of these cultivation experiments, I supposed *Leptospira icterohæmorrhagiæ* to be an obligatory or facultative anaerobe, because of its great facility for invading organs and multiplying in them. All attempts at cultivation failed as long as cultural conditions were employed which were calculated to produce anaerobiosis. The combination of conditions which I designated as aerotropic anaerobiosis[10] several years ago, and which was successfully used for the cultivation of the relapsing fever spirochetes, gave fairly good results when a suitable serum was used. But in the tubes to which a piece of fresh rabbit kidney was added, the cultures grew less luxuriantly and died out sooner than in the control tubes without the tissue. The simplicity of the cultural requirements of this organism was a surprise and led to the inference that the organism is an aerobe. When a number of subcultures of the Japanese, European, and American strains were cultivated at 37°C. in an anaerobic apparatus and another set without the exclusion of oxygen, excellent growth took place in all tubes where oxygen was accessible, while not a single organism could be found in the tubes kept in an anaerobic apparatus. The tubes were taken out of the anaerobic jar after 12 days and allowed to stand for several days at 37°C., but no new culture developed, probably because of the death of the organism during its stay in the anaerobic apparatus. *Leptospira icterohæmorrhagiæ*, therefore, has been shown to be an obligatory aerobe.

Detrimental Conditions Caused by Physical Hindrances to the Penetration of Oxygen into the Medium.

For obligatory aerobic bacteria a slant or plate agar or broth should be satisfactory, because most of this class of organisms grow in more

[10]Noguchi, *The Harvey Lectures*, 1915–16, 236.

or less discrete, often thick or elevated colonies on the surface of a solid medium. In broth the growth may be diffuse or superficial, forming a pellicle or thick scum. The use of a high layer agar or gelatin for the cultivation of such organisms means a waste of medium, since oxygen cannot penetrate the greater part of it. Since *Leptospira icterohæmorrhagiæ* is an obligatory aerobe, it follows that the addition of solid substances such as agar or gelatin, which must necessarily interfere with the entrance of oxygen into the medium, will be detrimental to the growth of the organism. The denser the concentration of agar or gelatin, the narrower is the zone to which oxygen can penetrate. The experiment summarized in Table IV shows the effect of different concentrations of agar or gelatin upon the culture. The gelatin and agar were made in a 0.5 per cent saline solution and adjusted to a slightly alkaline reaction.

TABLE IV.

Medium.	37°C.			26°C.		
	4 days.	7 days.	28 days.	4 days.	7 days.	28 days.
Gelatin (10 per cent) 4 cc.	−	−	−	−	−	−
Rabbit serum 1 cc. Gelatin (10 per cent) 3 cc.	+	−	−	<<+	−	−
Rabbit serum 1 cc. Gelatin (10 per cent) 1 cc. Ringer's solution 2 cc.	+	+++	+++	<+	+	+++
Rabbit serum 1 cc. Gelatin (10 per cent) 0.5 cc. Ringer's solution 2.5 cc.	+	+++	+++	<+	<+	+++
Agar (2 per cent) 4 cc.	−	−	−	−	−	−
Rabbit serum 1 cc. Agar (2 per cent) 3 cc.	+	++	<+	<<+	+	<<+
Rabbit serum 1 cc. Agar (2 per cent) 1 cc. Ringer's solution 2 "	+	++++	++++	+	++	++++
Rabbit serum 1 cc. Agar (2 per cent) 0.5 cc. Ringer's solution 2.5 "	++	++++	++++	+	++	++++

The experiment demonstrates the disturbing effect of gelatin when present in more than 7.5 per cent and of agar in more than 1.5 per cent. Agar, when added in proportions of 0.5 per cent and 0.25 per cent, considerably improved the cultural conditions. In this concentration it does not perceptibly hinder the penetration of oxygen into the medium and it offers to the spirochetes an ideal semisolid permeable substance. In this respect this particular culture medium is even better than a pure fluid medium. Gelatin, when added in proportions of 2.5 and 1.25 per cent, seems to have been neither beneficial nor detrimental to the growth of the culture.

Ordinary Culture Media and Leptospira icterohæmorrhagiæ.

It would be an economic advantage if a simpler method for the cultivation of this spirochete was devised. No culture was obtained, however, with any of the ordinary media, such as plain and 2 per cent glucose bouillon, Hiss serum water, litmus milk, plain and 2 per cent glucose agar, Loeffler's serum, glycerolated bouillon, and agar. A special bouillon medium formulated by Dr. Kligler was tried—1 per cent peptone, 0.5 per cent sodium phosphate, 0.1 per cent glucose, and 0.5 per cent sodium chloride—but without success. The presence of peptone, broth, casein, glucose, etc., instead of having a nutrient value for *Leptospira icterohæmorrhagiæ* in a suitable medium such as one containing the necessary amount of rabbit serum, seems to have a definite unfavorable influence upon the culture. The addition of a 10 per cent neutral solution of peptone 4.5 cc., to rabbit serum 1.5 cc., rendered the mixture unsuitable for a culture medium, as is not the case with indifferent diluents such as Ringer's solution, distilled water, or isotonic salt solution. Even the addition of approximately 1.5 per cent peptone suppressed growth to a marked degree. Bouillon or glucose bouillon are not good diluents for making up a culture medium for this organism.

Addition of Carbohydrates to Culture Media.

Akatsu,[11] while working in my laboratory, studied the action of various spirochetes upon many carbohydrates, but he did not find

[11] Akatsu, S., *J. Exp. Med.*, 1917, xxv, 375

definite fermentation phenomena in any of the organisms examined. With *Treponema mucosum* and *Treponema microdentium* a definite increase in the amount of acid was noticed. In the present experiment, the Japanese strain of *Leptospira icterohæmorrhagiæ* was cultivated in two sets of media of fourteen tubes each. In one set the media were made up of 1.5 cc. of rabbit serum, 1 cc. of a 10 per cent solution of carbohydrate, previously sterilized by filtration, 2.5 cc. of Ringer's solution, and 1 cc. of citrate plasma of the rabbit. In the other set 1 cc. of 2 per cent agar (melted) was used instead of the citrate plasma. The fourteen carbohydrates used in both sets were glucose, lactose, maltose, levulose, galactose, saccharose, dextrin, inulin, mannite, dulcite, isodulcite, arabinose, raffinose, and salicin. Tubes without carbohydrate and tubes also which were not inoculated with culture were used as controls.

The new generation of the culture became recognizable within a fortnight at 29°C. by the hazy layer at the top of the columns of culture media. The haze extended downwards from the surface to a depth of 1 to 1.5 cm. By examination under the dark-field microscope, the haze was found to represent dense diffuse colonies of actively multiplying spirochetes. The appearance of the haze was the same in the tubes containing the various carbohydrates as in the sugar-free control tubes. In the set where 1 per cent citrate plasma was used to form loose fibrin, the haze was less distinct but extended as far as 3 or 4 cm. below the surface, and the lower border was not sharply outlined as in the media with semisolid agar. The viability of the spirochetes was as great in the media containing carbohydrates as in those without carbohydrates. The reaction of the cultures failed to indicate any attack by the organism upon the carbohydrates. The reaction remained slightly alkaline to litmus paper as before cultivation, and was entirely comparable with the reaction in the spirochete-free controls.

Special attention was given to the detection of possible morphological modifications in the organisms grown in the presence of the carbohydrates, but none was recognized.

Influence of Temperature upon Cultivation.

Inada and his coworkers[2] found that *Leptospira icterohæmorrhagiæ* grows very well at room temperature, as it does at any temperature up to 37°C., but that at lower temperatures (20–25°C.) the organism survives longer than at 37°C.

I have cultivated three different strains of the spirochete at different temperatures. The results, as recorded in Table V, are self-explanatory. The media used consisted of rabbit serum 1 cc. + Ringer's solution 3 cc. + citrate plasma 1 cc. or 1.5 per cent agar 1 cc.

TABLE V.

Strains.	42°C.		37°C.		30°C.		25°C.		10°C.	
	7 days.	28 days.	7 days.	28 days.	7 days.	28 days.	7 days.	28 days.	7 days.	28 days.
Japanese.										
Plasma.	−	−	++++	++++	++++	++++	++++	++	++++	++
Agar.	−	−	+++	++++	+++	++++	+	<+	+	<+
European.										
Plasma.	−	−	++	+++	++	++	+	−	+	−
Agar.	−	−	++	+++	++++	++++	+	++	<<+	−
American No. 1.										
Plasma.	−	−	++	+++	++	+++	+	++	+++	++
Agar.	−	−	++	+++	++	++++	++	+++	++	+

The ability of the organism to multiply and remain active a long time at 10°C. is interesting from the epidemiological standpoint. It suggests that certain insects might serve as reservoirs of the virus.

Culture Media Recommended for Leptospira icterohæmorrhagiæ.

As a result of the experiments recorded on the relative nutrient value of various sera, the influence of reaction, oxygen tension, diluents, salts, and various other substances, I have formulated the following media:

A. Rabbit serum	1.5 parts.	
Ringer's solution	4.5 "	
Citrate plasma	1.0 part.	
Paraffin oil to cover the surface.		
B. Rabbit serum	1.5 parts.	
Ringer's solution	4.5 "	
2 per cent agar	1.0 part.	
Paraffin oil to cover the surface.		
C. Rabbit serum	1.5 parts.	Semisolid portion.
Ringer's solution	4.5 "	
2 per cent agar	1.0 part.	
After solidification add:		
Rabbit serum	1.5 parts.	Fluid portion.
Ringer's solution	4.5 "	
Paraffin oil to cover the surface.		

Growth usually begins much sooner in Medium A than in Medium B, but after a month more spirochetes will be found in B. For keeping up subcultures of various strains, Media A and B were simultaneously used in small test-tubes each containing 7 cc. of the composite medium.

For obtaining a large amount of culture, long necked flasks of medium capacity (50 to 100 cc.) were used. It was found best to fill the flasks with the medium to one-half or one-third their capacity and then to cover the surface with a very thin layer of paraffin oil. If the flasks are filled higher than this, oxygen becomes less accessible to the deeper part of the medium, especially when it contains agar. The use of a low layer semifluid medium (B) is based upon the fact, previously mentioned, that unrestricted multiplication of *Leptospira icterohæmorrhagiæ* takes place in such a medium on the surface stratum of 1 to 2 cm. Medium A is similarly semifluid, but the fibrin mass loosens and breaks up in time, especially by repeated withdrawal of the culture with pipettes, rendering the penetration of oxygen almost as easy as in a fluid medium. The flasks containing Medium A may therefore be filled half or two-thirds full, with a thin layer of paraffin oil.

Medium C seems to combine the advantages of Media A and B, the lower stratum being composed of Medium B, upon which, after solidification, is superimposed a mixture of rabbit serum and Ringer's solution (1:3). The medium is then inoculated and covered with a thin layer of paraffin oil. For subcultures, 0.1 or 0.2 cc. of a vig-

orously growing culture is pipetted on the surface of new culture media and then covered with paraffin oil.

D is a medium for acclimated strains. A fluid medium consisting of 1 part of horse or sheep serum and 3 parts of Ringer's solution or salt solution proved to be fairly suitable for strains which had become accustomed to the various media (A, B, C) during a period of several months.

SUMMARY AND CONCLUSIONS.

1. The presence of suitable animal or human serum is essential for the cultivation of *Leptospira icterohæmorrhagiæ*.

2. The nutrient value of serum is considerably reduced by heating to 60°C. for 30 minutes and is destroyed by boiling (100°C). Filtration through a Berkefeld filter does not diminish the nutrient value of the serum.

3. The cultural value of different animal sera varies considerably. It is entirely absent from the sera of the rat and the pig. The sera of the rabbit, horse, and goat are better suited for the growth of the organism than those of the guinea pig, sheep, donkey, or calf. Human serum is suitable, but not ascitic fluid.

4. Fresh or heated emulsions of the liver, kidney, heart muscle, or testicle of the normal guinea pig or rabbit have no cultural value for the organism. The same may be said of both the white and yolk of the hen's egg.

5. A luxuriant growth takes place in a medium of Ringer's solution to which more than 10 per cent of normal rabbit serum is added. There is only moderate growth with 5 per cent of serum, and none when less than 2 per cent is present. The use of an undiluted serum offers no advantage over a diluted one, provided the latter contains at least 10 per cent of serum. In the case of certain animal sera dilution seems to make them more suitable for cultivation purposes, owing perhaps to its reduction of their inherent alkalinity.

6. The tonicity of the culture medium has but little influence upon the growth and morphology of the organism. A medium containing distilled water as diluent or one containing 8 per cent sodium chloride seems to give identical results. The viability of the organism was

greatest in a medium in which Ringer's solution or isotonic salt solution was used as diluent.

7. The reaction of the medium is an important factor in the cultivation of the organism, which thrives most vigorously in a medium of which the reaction is slightly alkaline, not exceeding that of the serum. If the reaction is neutral, the growth is meager, and the culture is short lived. When the reaction of a medium becomes alkaline by the addition of a small amount of sodium hydroxide, or faintly acid by the addition of a little hydrochloric acid, no growth can take place.

8. *Leptospira icterohæmorrhagiæ* is an obligatory aerobe. Any hindrance to the access of oxygen constitutes an unfavorable factor in obtaining a culture.

9. The addition of carbohydrates to media has no perceptible effect upon the growth or morphology of the organism. The reaction of the media is not modified by their presence.

10. *Leptospira icterohæmorrhagiæ* grows at any temperature between 37° and 10°C., the optimum zone being 30–37°C. Growth proceeds more rapidly at 37°C. than at 30° or at 25°, but the cultures remain viable much longer at the latter temperatures. No growth takes place at 42°C.

11. Three different media are described for the cultivation of freshly isolated strains. After prolonged cultivation on these media a strain may be readily cultivated in a serum diluted with Ringer's or isotonic salt solution.